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ORIGINAL ARTICLE

Biological activity of intervenolin analogs with a phenyl substituent

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Intervenolin analogs with a phenyl substituent at the 2- or 3-position were synthesized. The compounds (3–11) showed weak or no inhibitory activity toward the growth of MKN-74 gastric adenocarcinoma cells, even in the presence or absence of the corresponding Hs738 stromal cells, whereas 2-substituted analogs exhibited selective anti-*Helicobacter pylori* activity. Introduction of a pendant side chain on the nitrogen alleviated their acute toxicity in mice. The 2-phenyl-substituted analogs are reasonable structural templates for structure–activity relationship studies toward the development of anti-*H. pylori* agents that do not affect human cells.
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INTRODUCTION

Tumor tissue comprises not only tumor cells, but also stromal cells, including fibroblasts and endothelial cells, that are normal cells.[1,2] In fact, extensive evidence indicates that stromal cells contribute largely to the growth of adjacent tumor cells,[3,4] as well as to tumor progression[5] and malignancy[6] (tumor–stromal interactions). Targeted cancer therapy using small-molecular-weight molecules that act on cancer-relevant proteinous proliferation regulators is nowadays frequently included in the cancer treatment regimen: these therapeutic agents almost exclusively influence the fate of the tumor cells without seriously damaging normal cells, and are thus less likely to produce side effects. Although this relatively new class of therapeutics has enjoyed great clinical success, the lability of cancer genes often leads to mutations of the molecular target, thereby increasing the risk of relapse of the treated cancer. Consequently, exploratory studies of chemotherapeutic agents with molecular targets of normal cell origin are in high demand.

In 2013, Kawada *et al.*[7] reported a quinolone-based natural product, intervenolin (**1**, Figure 1), produced by *Nocardia* sp. ML96-86F2 as a modulator of tumor–stroma interactions. Intervenolin inhibits the growth of gastric and colon tumor cell lines more potently in the presence of the corresponding stromal cells than in their absence. Our laboratory established a synthetic route to this compound by taking advantage of Suzuki–Miyaura coupling and thiocyanate–isothiocyanate rearrangement as key transformations to introduce a geranyl side chain and pendant iminodithiocarbonate moiety, respectively.[8] Both side chains are rare chemical motifs as substituents of quinolone-related compounds; the latter is particularly uncommon in natural products as a whole. The successful chemical synthesis of intervenolin enabled detailed biological investigations, including *in vivo* antitumor tests and structure–activity relationship (SAR) studies.[9] Indeed, the first SAR study revealed that (1) substituents at the 2-position have a large impact on the biological activity, (2) a substituent on the nitrogen is beneficial for reducing acute toxicity and (3) many of the synthetic intervenolin analogs exhibit highly selective anti-*Helicobacter pylori* activity over a variety of pathogenic bacteria: inspired by the reported work on natural quinolones,[10] the antibacterial activity was investigated. On the other hand, SAR information on the 3-position has not been collected. More importantly, separation of the antiproliferative activity and anti-*H. pylori* activity for lead-generation purposes would help to avoid undesirable side effects. In the above-mentioned SAR study, an intervenolin analog with a 4-fluorophenyl group at the 2-position (**2**, Figure 1) exhibited no growth-inhibitory activity (IC_{50}: $>100\ \mu g\ ml^{-1}$) toward MKN-74 gastric adenocarcinoma cells, irrespective of the presence or absence of stromal cells (Hs738), whereas it exhibited moderate anti-*H. pylori* activity (MIC: $0.2\ \mu g\ ml^{-1}$). These results prompted us to carry out further SAR studies of intervenolin analogs with aromatic substituents (Scheme 1), and the results are disclosed herein. Initially, compounds with a phenyl group at the 2-position were evaluated, followed by investigation of compounds with a phenyl group at the 3-position instead of the original methyl group. For the latter analogs, the side chain at the 2-position was fixed to C8 that was demonstrated to be the preferred length in a previous SAR study.[9]

RESULTS AND DISCUSSION

Synthesis

The synthesis was accomplished uneventfully according to the reported protocol[9] (Scheme 1) starting from aniline derivatives equipped with a ketone functionality that was converted to the corresponding amide with installation of the structural element for the 2-substituent.

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Subsequent annulation constructed the requisite quinoline core, followed by functionalization of the nitrogen, if needed.

Antiproliferative activity toward gastric cancer cells (MKN-74) under cocultured conditions with gastric stromal cells (Hs738) and monocultured conditions

We first examined the activity of each synthesized intervenolin analog (**3–11**) as a modulator of tumor–stromal interactions (Table 1), as the growth-inhibitory activity toward MKN-74 gastric tumor cells in the presence or absence of Hs738 stromal cells was evaluated as in the previous SAR study. For 2-phenyl-substituted analogs **3–5**, no antiproliferative activity was observed under either cocultured or monocultured conditions, consistent with the results of the 2-(4'-flurophenyl)-substituted compound (**2**). The tendency for substituents at the 1-position to reduce acute toxicity (i.v., single dose), which was observed in the previous study, was also observed in this case (compare **2** with **3** and **5**). Introduction of a phenyl group instead of a methyl group at the 3-position (**6–11**) had detrimental effects regardless of the substituent on the nitrogen. Further SAR studies are needed to clarify whether the results were due to the bulkiness of the benzene ring.

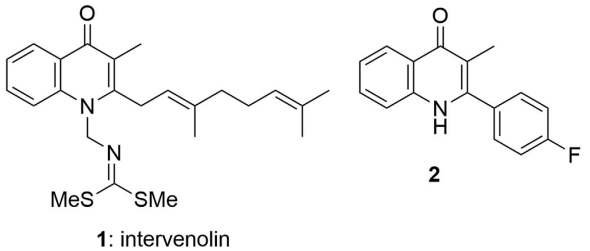
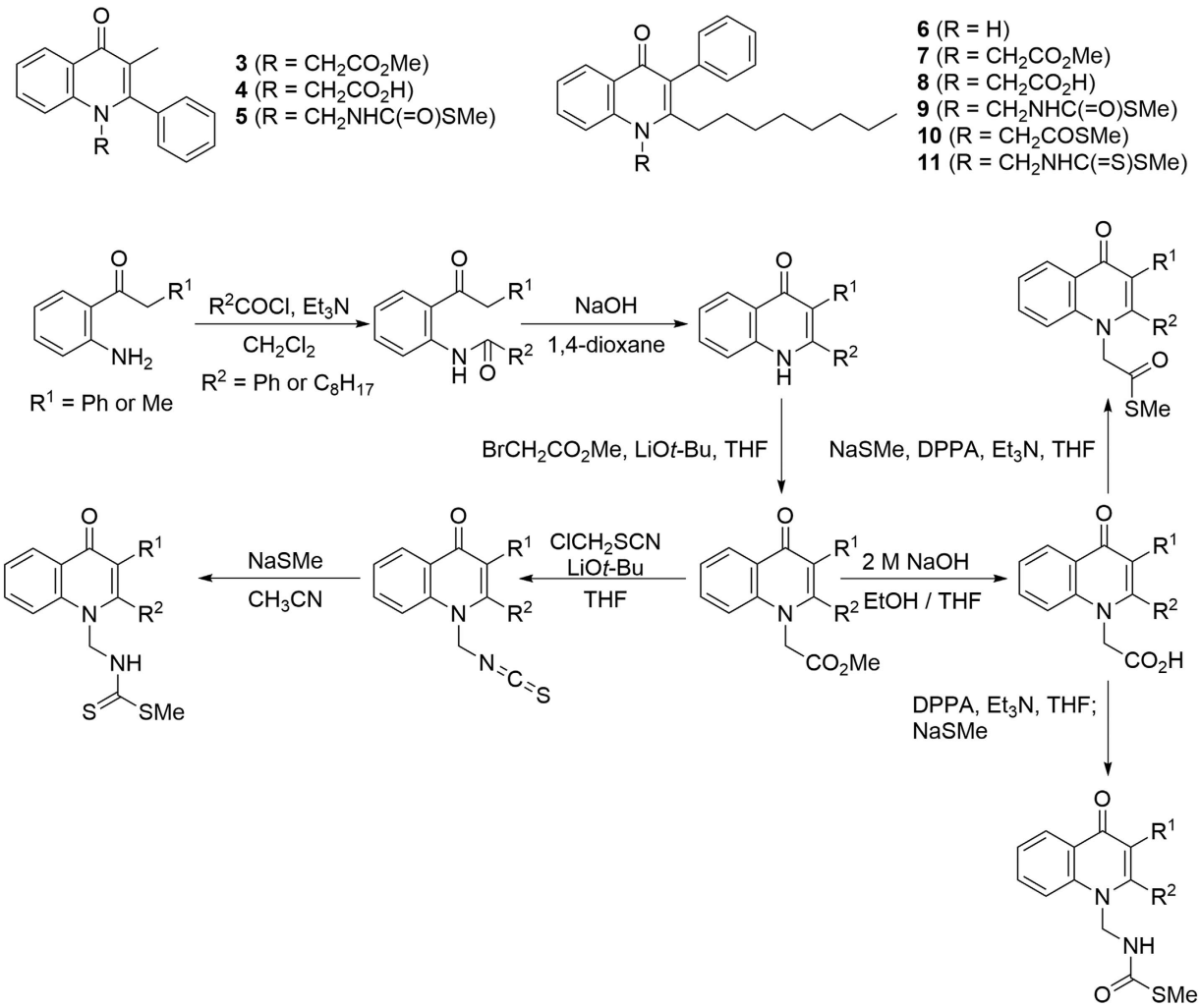


Figure 1 Intervenolin and an analog.

Anti-*H. pylori* activity

In contrast to the data regarding their properties as modulators of tumor–stromal interactions shown in Table 1, the intervenolin analogs with the phenyl functionality at the 2-position (**3–5**) displayed anti-*H. pylori* activity (Table 2). In particular, the potency of **3** containing a methoxycarbonylmethyl moiety at the 1-position exhibited the best anti-*H. pylori* activity among the compounds synthesized herein; fourfold less potent toward JCM 12093 strain (MIC: 0.25 $\mu g\,ml^{-1}$) than the positive control used in this study, clarithromycin (MIC: 0.0625 $\mu g\,ml^{-1}$). The potency of **2** is cited from the previous study in Table 2 that was twofold less potent than clarithromycin (MIC: 0.0078 $\mu g\,ml^{-1}$).[9] It is noteworthy that this class of compounds (**3–5**) exhibited limited antibacterial activity toward other species, such as



Scheme 1 Intervenolin analogs in this study, and outline of the synthesis.

Campylobacter coli, *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli*, clearly demonstrating that these analogs maintain high selectivity toward *H. pylori*, a characteristic property of some intervenolin analogs synthesized in the previous study. As expected, the antibacterial activity was independent of the growth-inhibitory activity. In fact, 3-phenyl-substituted analogs (**6–11**) again exhibited limited anti-*H. pylori* potency (that is, MIC: 4–64 μg ml^{-1}); this mode of substitution is not suitable for molecular design aimed at pursuing both biological activities.

EXPERIMENTAL PROCEDURES

General

NMR was recorded on JEOL (Akishima, Japan) ECS-400 and ECA-600 spectrometers. Chemical shifts of protons are reported in ppm downfield from TMS or are referenced to residual protium in the NMR solvent ($CDCl_3$: δ 7.26 ppm, CD_3OD: δ 3.30 ppm, DMSO-d_6: 2.49). For ^{13}C NMR, chemical shifts were reported in ppm downfield from TMS or are relative to the NMR solvent ($CDCl_3$: 77.0 ppm, CD_3OD: δ 49.0 ppm, DMSO-d_6: 39.7 ppm) as an internal reference. NMR data are reported as follows: chemical shifts, multiplicity (s: singlet, d: doublet, dd: doublet of doublets, t: triplet, m: multiplet, br: broad), coupling constant (Hz) and integration. HR-MS (electrospray ionization (ESI) − time-of-flight (+)) were measured on Thermo Fisher Scientific (Yokohama, Japan) LTQ Orbitrap XL. Unless otherwise noted, materials were purchased from commercial suppliers and were used without purification.

Table 1 Effects of the synthetic intervenolin derivatives on cocultured MKN-74 cells and Hs738 cells

	In vitro IC_{50} (μg ml^{-1})		
Compound	*Cocultured*	*Monocultured*	*In vivo MTD (mg kg^{-1})*
1	0.52	3.9	>50
2	>10	>10	6.25[a]
3	>10	>10	>50
4	>10	>10	NT
5[b]	>10	>10	>50
6	>10	>10	NT
7	5.9	>10	>50
8	>10	>10	>50
9	4.3	9.3	>50
10	>10	>10	NT
11	5.9	~10	NT

Abbreviations: MTD, maximum tolerated dose; NT, not tested.
[a]Data from Abe *et al.*[9]
[b]Contains a small amount of impurity.

Synthesis

Intervenolin analogs were synthesized according to the reported procedure. Characterization data for **3–11** (novel compounds) are listed below. A copy of NMR charts is in Supplementary Material.

*Methyl 2-(3-methyl-4-oxo-2-phenylquinolin-1(4H)-yl)acetate (**3**).* ^1H NMR (500 MHz, $CDCl_3$) δ 8.56 (1H, dd, J=8.2, 1.6 Hz), 7.64 (1H, m), 7.55–7.51 (3H, m), 7.40 (1H, m), 7.31–7.28 (2H, m), 7.19 (1H, d, J=8.5 Hz), 4.61 (2H, s), 3.71 (3H, s), 3.62–3.51 (2H, m), 1.84 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.8, 168.6, 150.6, 140.5, 135.0, 132.3, 129.5, 129.4, 128.3, 127.4, 125.1, 123.4, 118.6, 114.6, 52.8, 50.3, 13.4; HR-MS (ESI) Anal calcd for $C_{19}H_{18}NO_3$ *m/z* 308.1287 $[M+H]^+$, found 308.1279.

*2-(3-Methyl-4-oxo-2-phenylquinolin-1(4H)-yl)acetic acid (**4**).* ^1H NMR (500 MHz, DMSO-d_6) δ 8.28 (1H, dd, J=7.9, 1.6 Hz), 7.72 (1H, m), 7.62–7.55 (4H, m), 7.41 (1H, m), 7.38–7.22 (2H, br), 1.66 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 176.0, 169.5, 150.9, 140.4, 134.7, 132.1, 129.4, 129.3, 128.0, 125.7, 124.2, 123.1, 116.6, 116.5, 50.1, 13.1; HR-MS (ESI) Anal calcd for $C_{18}H_{16}NO_3$ *m/z* 294.1130 $[M+H]^+$, found 294.1126.

*S-Methyl((3-methyl-4-oxo-2-phenylquinolin-1(4H)-yl)methyl)carbamothioate (**5**).* ^1H NMR (500 MHz, $CDCl_3$) δ 8.66 (1H, br), 8.12–8.08 (1H, br), 7.59–7.50 (5H, m), 7.40 (1H, m), 7.23 (1H, d, J=8.5 Hz), 7.17 (1H, br), 4.61 (2H, s), 2.47 (3H, s), 1.47 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.8, 169.9, 151.1, 140.2, 134.6, 132.4, 129.8, 129.5, 128.2, 126.7, 125.0, 124.0, 118.7, 115.2, 53.6, 22.6, 13.2; HR-MS (ESI) Anal calcd for $C_{19}H_{19}NO_2S$ *m/z* 339.1167 $[M+H]^+$, found 339.1155.

*2-Octyl-3-phenylquinolin-4(1H)-one (**6**).* ^1H NMR (400 MHz, $CDCl_3$) δ 8.34 (1H, d, J=8.3 Hz), 7.41 (1H, m), 7.29–7.19 (6H, m), 7.15–7.10 (1H, m), 2.30 (2H, m), 1.42 (2H, m), 1.19–1.03 (10H, m), 0.87 (3H, t, J=7.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.9, 152.4, 139.9, 136.4, 131.1, 131.0, 128.1, 126.8, 125.4, 124.6, 123.0, 121.6, 118.7, 32.3, 31.7, 29.2, 29.1, 28.9, 22.5, 14.0; HR-MS (ESI) Anal calcd for $C_{23}H_{18}NO$ *m/z* 334.2171 $[M+H]^+$, found 334.2167.

*Methyl 2-(2-octyl-4-oxo-3-phenylquinolin-1(4H)-yl)acetate (**7**).* ^1H NMR (400 MHz, $CDCl_3$) δ 8.48 (1H, dd, J=8.1, 1.5 Hz), 7.63 (1H, m), 7.44–7.32 (4H, m), 7.28–7.25 (3H, m), 4.95 (2H, s), 3.83 (3H, s), 2.54 (2H, br), 1.50 (2H, m), 1.28–1.08 (10H, m), 0.86 (3H, t, J=7.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.7, 168.6, 151.8, 141.1, 136.6, 132.3, 130.6, 128.4, 127.6, 127.2, 126.2, 124.7,

Table 2 Antibacterial activities of the synthetic intervenolin derivatives (MIC: μg ml^{-1})

	Helicobacter pylori JCM	*H. pylori JCM*	*Campylobacter coli JCM*	*Staphylococcus aureus*	*Enterococcus faecalis JCM*	*Escherichia coli*
Compound	*12093*	*12095*	*2529*	*FDA209P*	*5803*	*K-12*
1	0.125	0.0625	32	128	>128	>128
2[a]	0.0156	0.0156	NT	128	>128	128
3	0.25	0.5	64	128	>128	128
4	1	2	64	128	>128	128
5[b]	0.5	1	64	128	>128	128
6	32	64	>128	128	128	>128
7	4	8	>128	4	>128	128
8	32	64	128	>128	>128	128
9	8	16	64	>128	>128	>128
10	32	32	64	128	>128	>128
11	16	16	64	>128	>128	>128
CAM	0.0625	0.0312	4	<0.125	0.25	16

Abbreviations: CAM, clarithromycin; NT, not tested.
[a]Data from Abe *et al.*[9]
[b]Contains a small amount of impurity.

123.5, 114.4, 53.0, 48.5, 31.6, 31.5, 29.4, 28.88, 28.86, 28.7, 22.5, 14.0; HR-MS (ESI) Anal calcd for $C_{26}H_{32}NO_3$ *m/z* 406.2382 $[M+H]^+$, found 406.2374.

*2-(2-Octyl-4-oxo-3-phenylquinolin-1(4H)-yl)aceticacid (**8**).* ^{1}H NMR (400 MHz, CD_3OD) δ 8.35 (1H, dd, $J=8.1$, 1.5 Hz), 7.72 (1H, m), 7.66 (1H, d, $J=8.6$ Hz), 7.47–7.35 (4H, m), 7.26–7.24 (2H, m), 5.04 (2H, br), 2.62 (2H, br), 1.56 (2H, m), 1.29–1.06 (10H, m), 0.87 (3H, t, $J=6.9$ Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 178.1, 156.3, 142.9, 138.2, 133.6, 132.0, 129.6, 128.4, 127.3, 126.9, 125.2, 124.9, 117.7, 51.6, 32.9, 32.7, 30.5, 30.0, 29.6, 29.4, 23.6, 14.4; HR-MS (ESI) Anal calcd for $C_{25}H_{30}O_3$ *m/z* 392.2226 $[M+H]^+$, found 392.2215.

*S-Methyl((2-octyl-4-oxo-3-phenylquinolin-1(4H)-yl)methyl)carbamothioate (**9**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (1H, m), 7.63 (1H, m), 7.54 (1H, d, $J=8.5$ Hz), 7.36–7.28 (4H, m), 7.13 (2H, d, $J=6.9$ Hz), 6.92 (1H, br), 5.71 (2H, s), 2.69 (2H, m), 2.37 (3H, s), 1.48 (2H, m), 1.27–1.08 (10H, m), 0.86 (3H, t, $J=7.3$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.7, 168.7, 152.3, 140.1, 132.6, 130.5, 128.4, 127.6, 126.0, 124.5, 123.7, 115.0, 52.2, 31.7, 31.1, 29.8, 29.4, 28.9, 28.8, 22.6, 14.1, 12.4; HR-MS (ESI) Anal calcd for $C_{26}H_{33}N_2O_2S$ *m/z* 437.2263 $[M+H]^+$, found 437.2253.

*S-Methyl 2-(2-octyl-4-oxo-3-phenylquinolin-1(4H)-yl)ethanethioate (**10**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 (1H, m), 7.63 (1H, m), 7.45–7.32 (4H, m), 7.29–7.25 (3H, m), 5.07 (2H, br), 2.54 (2H, m), 2.36 (3H, s), 1.50 (2H, m), 1.27–1.06 (10H, m), 0.86 (3H, t, $J=7.1$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.5, 176.7, 151.9, 141.1, 136.5, 132.3, 130.6, 128.4, 127.6, 127.2, 126.2, 125.0, 123.7, 114.8, 56.0, 31.6, 31.5, 29.3, 29.0, 28.8, 28.6, 22.5, 14.0, 11.4; HR-MS (ESI) Anal calcd for $C_{26}H_{32}NO_2S$ *m/z* 422.2154 $[M+H]^+$, found 422.2145.

*Methyl((2-octyl-4-oxo-3-phenylquinolin-1(4H)-yl)methyl)carbamodithioate (**11**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 (1H, m), 8.03 (1H, br), 7.66 (1H, m), 7.47 (1H, d, $J=8.6$ Hz), 7.40–7.26 (4H, m), 7.10 (1H, m), 6.13 (2H, br), 2.66 (3H, s), 2.60 (2H, m), 1.52 (2H, m), 1.27–1.06 (10H, m), 0.86 (3H, t, $J=7.1$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.1, 176.9, 152.2, 140.2, 136.1, 132.9, 130.4, 128.4, 127.4, 127.3, 125.7, 124.6, 124.0, 115.2, 58.1, 31.7, 31.5, 29.5, 29.4, 28.9, 28.7, 22.6, 18.2, 14.1; HR-MS (ESI) Anal calcd for $C_{26}H_{33}N_2OS_2$ *m/z* 453.2034 $[M+H]^+$, found 453.2024.

Cells and reagents

Human gastric adenocarcinoma MKN-74 cells were obtained from the RIKEN cell bank (Tsukuba, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Sigma, Tokyo, Japan), 100 units per ml penicillin G (Life Technologies, Carlsbad, CA, USA) and 100 μg ml^{-1} streptomycin (Life Technologies) at 37 °C with 5% CO_2. Hs738 human gastric stromal cells (CRL-7869) were obtained from ATCC (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS, 100 units per ml penicillin G, 100 μg ml^{-1} streptomycin, ITH (5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, and 1.4 μM hydrocortisone) and 5 ng ml^{-1} basic-fibroblast growth factor (Pepro Tech, Rocky Hill, NJ, USA) at 37 °C with 5% CO_2 as described previously.[11]

Green fluorescence protein (GFP) transfection

MKN-74 cells (2×10^5) were cultured in 6-well plates in 10% FBS–DMEM for 2 days. The medium was replaced by 800 μl OPTI-MEM (Life Technologies), and then 200 μl OPTI-MEM containing 1 μg pEGFP-C1 vector (BD Biosciences, Franklin Lakes, NJ, USA), 4 μl Lipofectamine Reagent (Life Technologies) and 6 μl PLUS Reagent (Life Technologies) was added to the cells. After 3 h of incubation, 1 ml of 20% FBS-OPTI-MEM was added to each well and the cells were cultured for 24 h. Stably transfected cells were selected with 400 μg ml^{-1} G418 (Promega, Tokyo, Japan).

Cell growth and coculture experiment

For coculture experiments, Hs738 cells were first inoculated in 96-well plates at 5×10^3 cells per well in 0.1 ml DMEM supplemented with 1% D-FBS and ITH. Test samples were added to the wells and Hs738 cells were cultured for 2 days. Then, 10 μl of MKN-74 cell suspension (5×10^3 cells) in serum-free DMEM was inoculated onto a monolayer of Hs738 cells and the cells were cultured further for 3 days. For monoculture of MKN-74 cells, only assay medium with test samples was first incubated for 2 days, and then MKN-74 cells were inoculated as described above and further cultured for 3 days. The growth of MKN-74 cells was determined by measuring GFP fluorescence intensity (excitation at 485 nm and emission at 538 nm) by lysing the cells in 10 mM Tris-HCl, 150 mM NaCl, 0.9 mM $CaCl_2$ and 1% Triton X-100.

Acute toxicity *in vivo*

Female ICR mice, 4-week old, were purchased from Charles River Breeding Laboratories (Yokohama, Japan) and maintained in a specific pathogen-free barrier facility according to our institutional guidelines. Samples were injected i.v. into mice. Over 2 weeks of observation, half of the dose that caused death or severe toxic effect was designated as the maximum tolerated dose in this study.

Antimicrobial effect

H. pylori JCM12093, *H. pylori* JCM12095, *C. coli* JCM2529, and *E. faecalis* JCM5803 were purchased from Japan Collection of Microorganisms (Tsukuba, Japan). *S. aureus* FDA209P and *E. coli* K-12 were from the in-house collection of the Institute of Microbial Chemistry. The MICs were determined by using the microdilution broth method in 96-well plates. Twofold serial dilutions of the test samples were prepared with DMSO. The dilution series were initially prepared at 200-fold final concentrations from this stock to provide a final test range of 128–0.001 μg ml^{-1}. Then, 50 μl of each dilution and 50 μl of test organisms ($2–9\times10^4$ CFUs per ml) were dispensed into each well. The MIC was defined as the lowest concentration of compound at which no visible growth could be detected. The test organisms, medium and culture conditions were as follows: *H. pylori* JCM12093 and *H. pylori* JCM12095 and *C. coli* JCM2529 were cultured in a Brain Heart Infusion Broth medium (BD Biosciences) with 10% FBS at 37 °C for 144 h under microaerobic conditions of 85% N_2, 5% O_2 and 10% CO_2; *S. aureus* FDA209P and *E. coli* K-12 were cultured in a nutrient broth medium consisting of 1% polypentone (Nihon Pharmaceutical, Tokyo, Japan), 1% fish extract (Kyokuto, Tokyo, Japan) and 0.2% NaCl for 18 h at 37 °C. *E. faecalis* JCM5803 was cultured in a Heart Infusion Broth medium (BD Biosciences) at 37 °C for 18 h.

CONCLUSIONS

Intervenolin analogs with a phenyl substituent at the 2- or 3-position were synthesized. The compounds (**3–11**) showed weak or no inhibitory activity toward the growth of MKN-74 gastric cancer cells, even in the presence or absence of the corresponding Hs738 stromal cells. On the other hand, 2-substituted compounds had selective anti-*H. pylori* activity with reasonable potency. Further SAR studies focused on 2-aromatic analogs to generate anti-*H. pylori* lead compounds, mechanistic study on antiproliferative and anti-*H. pylori* activities and investigation on antibacterial activity toward drug-resistant strains of *H. pylori* are underway and will be reported in due course.

DEDICATION

Dedicated to the late Professor Hamao Umezawa for his great achievement on development of antibiotics including kanamycin.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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